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**14. ABSTRACT**

The purpose of the research done has been to determine the mechanism for overexpression of JAB1 through transcription factor analysis and FISH as well as the role of JAB1 in Herceptin resistance. The major findings thus far are 1) The promoter region of JAB1 key transcription factors were identified that may drive JAB1 expression, C/EBP alpha, C/EBP beta, and STAT3. We found that STAT3 together with C/EBP alpha and beta greatly increase JAB1 transcription. We have evidence that both STAT3 and C/EBP bind to the JAB1 promoter. Further, that SRC is one key upstream activator of JAB1 transcription. 2) We have performed FISH on a number of breast cancer cell lines and patient fine needle aspirations and have seen amplification of the JAB1 locus 3) JAB1 overexpression confers resistance to Herceptin in breast cancer cell lines SKBR3 and BT474, and inhibition of JAB1 increased the efficacy of Herceptin mediated G1 arrest and p27 accumulation. Additionally, JAB1 was found to correlate with Herceptin resistance in human breast cancer tissue samples. We have completed nearly all of the tasks listed for this time period and are on track as indicated in the SOW.

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**Terry J. Johnson**
**The Regulation of JAB1 and Its Role in Breast Cancer**
**DOD-Predoctoral BC050913**

Progress report year 2

## Introduction

The purpose of this project is to determine the key transcription factors that are driving JAB1 expression, determine if JAB1 copy number is amplified in breast cancer, and to evaluate whether JAB1 overexpression in breast cancer plays a role in resistance to Herceptin treatment and whether inhibition of JAB1 will enhance sensitivity to treatment. In the past year, we have accomplished many of the tasks laid out in the Statement of Work (SOW). Tasks 1A-C, Tasks 2A, and Task 3A-C have been completed. We have begun working on Tasks 2B. The data from these tasks are shown in this annual update.

## Body

Task 1 of this proposal focused on characterizing the JAB1 promoter and its transcriptional regulation. We have identified the transcriptional start site and putative transcription factor binding sites of the JAB1 gene. In order to isolate and characterize the jab1 gene and its 5'-flanking region, we began by determining the transcriptional start site by primer extension analysis. It was found to be 91 bp upstream of the ATG translational start codon by primer extension analysis. A TATA box and CCAAT box was found within 77bp of this start site. Putative promoter regions were located using Proscan software and a series of 5' deletion mutants were constructed. PCR products varying from 32 to 2000 bp were subcloned into the pGL3 luciferase vector. The minimal promoter was found to be 472 base pairs upstream of the transcriptional start site. The region between –472 and –344 bp upstream of the ATG was essential for transcription. this region contains GATA and C/EBP consensus sequence.

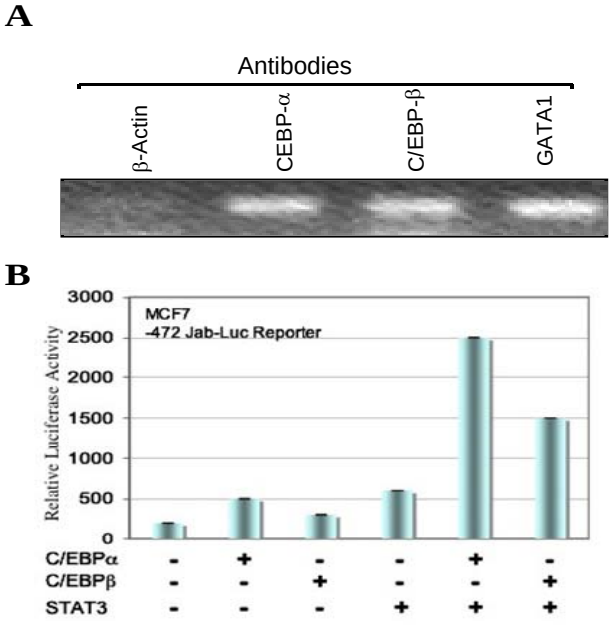


**Figure 1.** *Binding of C/EBPα, C/EBPβ, and GATA1 to the jab1 promoter and enhanced transactivation by STAT3* (A) Identification of proteins in complexes by ChIP assays. Antibodies for B-actin, C/EBP α, C/EBPβ, or GATA were immunoprecipitated from the sonicated lysates of MCF7 cells. After reversing the cross-linking, DNA was precipitated and PCR was performed using primers to amplify the -472/344 promoter DNA. The PCR products were then run on 2% agarose gels and visualized by ethidium bromide staining. (B) STAT3 enhances C/EBPα and C/EBPβ induction of *jab1* transcriptional activity. MCF7 cells were transfected the -472 Jab1-Luc construct along with STAT3, C/EBPα, C/EBPβ alone or in combination.

We have successfully identified the transcription factors that drive JAB1 transcription. We used site directed mutagenesis to mutate the DNA-response elements GATA and C/EBP in the region –472 to -344. These mutants were sub-cloned into the pGL3-basic vector and tested for luciferase activity in MCF7 cells. Mutation of both reduced the promoter activity. C/EBPα, C/EBPβ, and GATA1 were found to bind directly to the JAB1 promter by electrophorteic mobility shift assays (EMSAs) and by chromatin immunoprecipitation as shown in Figure 1A. Additionally, we observed that while C/EBP alpha and beta were able to transactivate the promoter, addition of STAT3 greatly enhanced this activity (Figure 1B).

It is known that STAT3 can bind directly with C/EBP and activate transcription. We questioned whether STAT3 was competitively binding directly to the promoter as there is a putative binding site in the same location as the C/EBP binding site that was not picked up by the MatInspector software (Figure 2A). By performing CHIP with the STAT3 antibody and transfecting increasing amounts of C/EBP beta-2 it is clear that STAT3 is able to bind to the promoter and this is enhanced by C/EBP beta-2 (Figure 2B). However, increasing the C/EBP beta-2 concentration led to competition for binding to the JAB1


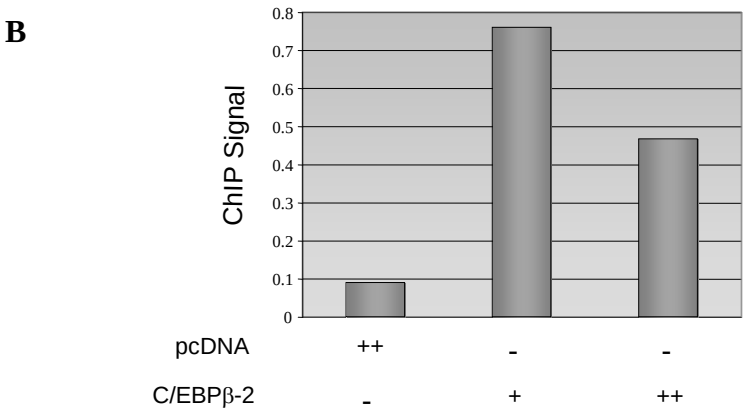


Figure 2. *STAT3 competes for binding with C/EBP to the jab1 promoter* (A) A potential STAT binding element (TTN5AA consensus sequence) overlaps the C/EBP binding site (B) STAT3 is able to bind to the *jab1*promoter, this binding was enhanced upon addition of C/EBPβ-2 but this was reduced when more C/EBPβ-2 was added. MDA-MB-231 cells were transfected with either control or C/EBPβ–2 (2ug or 5 ug) and collected for ChIP assays using an antibody to STAT3 and primers for the -472 to -375 region of the *jab1* promoter. The bands intensities were quantified and normalized to the input.

promoter. Further, we analyzed whether inhibition of STAT3 by siRNA or dominant negatives would affect JAB1 transcription. In Figure 3A transfection of either the phosphorylation mutant DN-STAT3 Y-F or the DNA binding mutant DN-STAT3 EEE/VV greatly reduced JAB1 transcription (A) and protein levels (B). Additionally, inhibtition of STAT3 by siRNA reduced JAB1 transcription (Fig. 4A) and protein levels (Fig. 4B). Next we questioned whether the non-receptor tyrosine kinase Src which is an activator of STAT3 activity was capable of regulating JAB1 transcription. We found that inhibition of Src by siRNA reduced JAB1 transcription (Fig 4A).

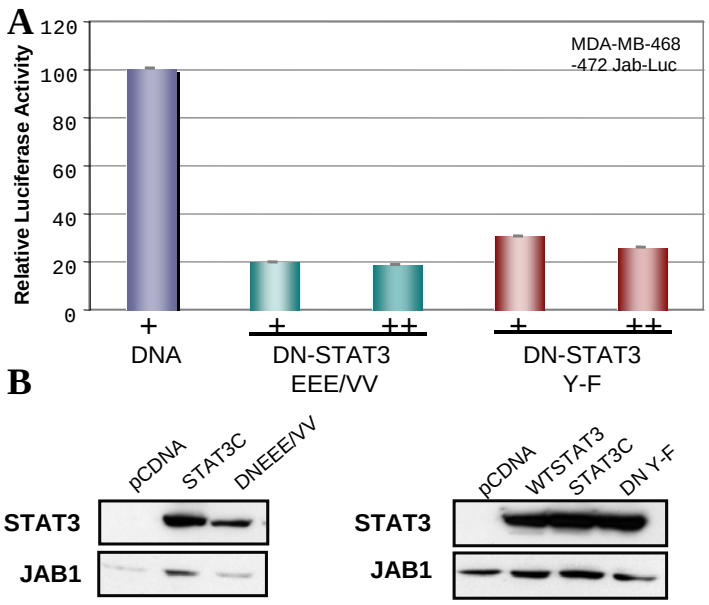


**Figure 3.** *Inhibition of STAT3 by dominant negatives reduced jab1 promoter activity.* MDA-MB-468 cells were co-transfected with either pCDNA control, STAT3C (constitutively active), DN-STAT3 EEE/VV, or DN-STAT3 Y-F with the -472 Jab1-Luc construct and subjected to luciferase reporter assays (A) and WB (B). Inhibition of *jab1* promoter activity and protein levels was seen upon addition of either dominant negative.

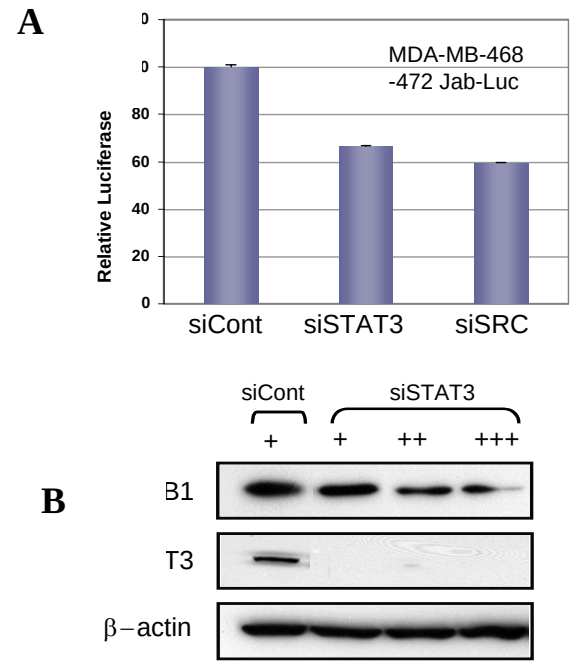


**Figure 4.** *Inhibition of STAT3 and SRC reduces jab1 promoter activity*
MDA-MB-468 cells were co-transfected with siRNA for STAT3 or SRC along with the -472 Jab1-Luc construct and subjected to luciferase reporter assays (A) or WB (B). Inhibition of STAT3 and SRC reduced *jab1* promoter activity and siSTAT3 reduced JAB1 protein levels.

We have successfully completed the goals of Task1. We will further identify the pathway that is driving transcription of JAB1 through these transcription factors. When this is completed we will submit this work for publishing.

Task 2 was to evaluate JAB1 genetic changes by fluorescence in situ hybridization (FISH). We have successfully performed FISH on fine needle aspirations (FNAs) from primary breast cancers using probes for choromosome 8 (CEP8) and jab1. To complete this task, we will perform FISH on more FNAs and will further evaluate whether outcome can be determined by JAB1 detection, proving that JAB1 would be a valuable prognostic marker.

Task 3 of this proposal aimed to investigate the role of JAB1 in resistance to Herceptin (Trastuzumab-Ttzm) treatment. We have successfully shown that overexpression of JAB1 in breast cancer cells leads to Herceptin-resistance through inhibition of p27 as projected in Task 3A. To determine whether overexpression of JAB1 provides a protective effect against Herceptin, we used HER2 overexpressing breast cancer cells, SKBR3 and BT474. These cells were transduced with a doxycyclin-regulated (Tet-Off system) adenovirus (Ad-JAB1) and were further treated with Herceptin (10ug/mL) in the absence (-) or presence (+) of doxycycline (1ug/mL) for 48 h, followed by western blotting and flow cytometry analysis. Herceptin treatment led to an increase in both p27 protein levels and G1 arrest that was inhibited by the overexpression of JAB1.

We concluded Task 3B and successfully show that inhibition of JAB1 increases the ability of Herceptin to induce G1 arrest. Herceptin resistant cells BT474 C#5 and C#6 were obtained from Timothy Kute. These cells are able to grow similar to the parental cell line, BT474, in the presence of Herceptin. After treatment with Herceptin they have lower phospho-AKT but no increase in nuclear p27. We stabily expressed shLUC and shJAB1 using a retroviral system in the C5 and C6 cells. Inhibition of JAB1 by shRNA in these cells (Figure 5A) increased the ability of Herceptin to induce G1 arrest (Figure 5B) and p27 accumulation (Figure 5C).

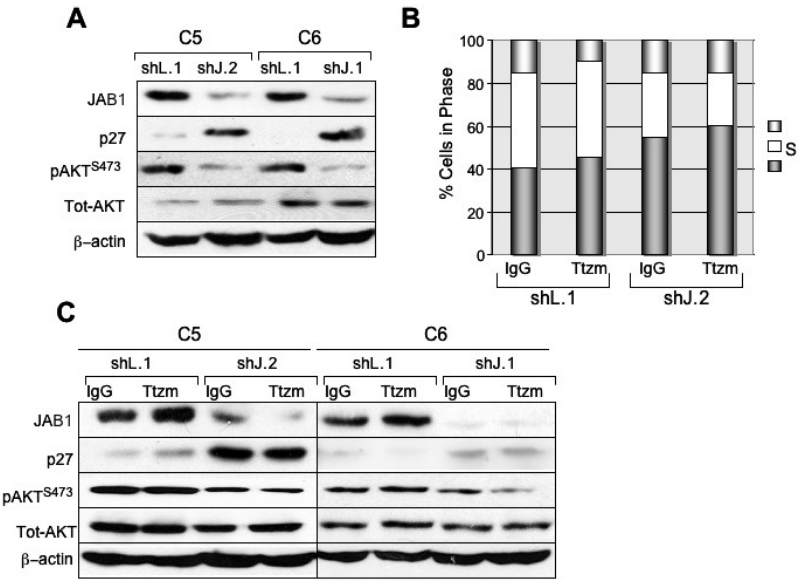


**Figure 5.** *Silencing JAB1 increased sensitivity to trastuzumab-mediated p27 accumulation and G1 arrest in resistant cells.* (*A*) Western blot analysis of JAB1 and p27 in shLUC (shL) and shJAB1 (sh-J) stable C5 and C6 clones demonstrating that inhibition of JAB1 effectively increased p27 levels in C5 and C6. Inhibition of JAB1 increased Herceptin )trastuzumab-Ttzm) induced G1 arrest as revealed by propidium iodide staining (*B*) and increased p27 accumulation as revealed by immunoblotting (*C*).

Further, inhibition of JAB1 reduced cellular proliferation compared to the shLUC clones and was sensitive an additional reduction in cell growth in both the C5 (Figure 6A) and C6 (Figure 6B) clones after Herceptin treatment. A wound healing assay showed that inhibition of JAB1 reduced the motility of the Herceptin resistant clone C6 that was sensitive to Herceptin (Figure 6C and 6D).

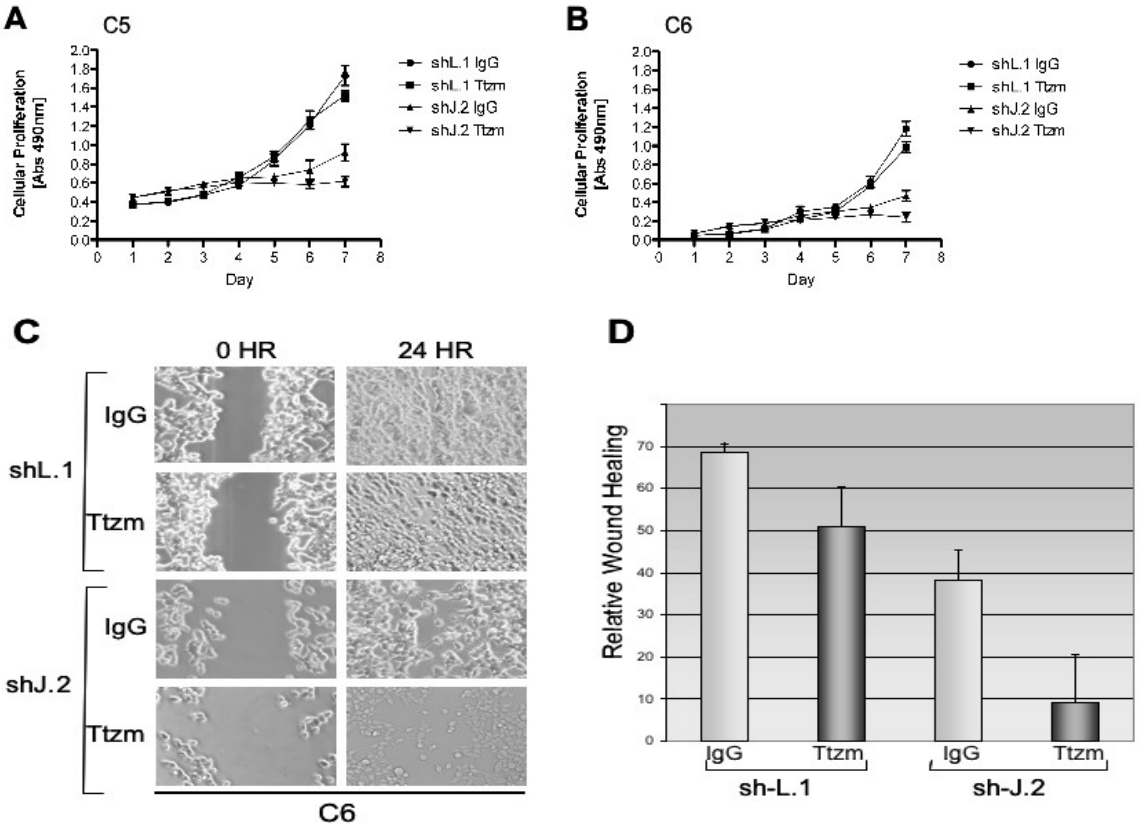


**Figure 6.** *Inhibition of JAB1 sensitized resistant cells to trastuzumab-mediated growth arrest and reduced motility.* Silencing JAB1 inhibited cellular proliferation that was further augmented by trastuzumab in the C5 (*A*) and C6 (*B*) stable cell lines. An MTS assay to detect cellular proliferation was done each day for 7 days after shLUC and shJAB1 stable C5 and C6 clones were treated with trastuzumab. (*C*) Trastuzumab inhibited motility in the C6 shJAB1 but not the resistant C6 shLUC. After scratch wounding, C6 shLUC and shJAB1 stable cells were cultured in the presence of IgG or trastuzumab. Phase contrast microscopy was carried out at 0 and 24 h to record the relative rate of wound healing, determined by the number of cells in the wound area after 24 h. (D) Quantification of the wound healing assays (C).

We also completed Task 3C and discovered that expression of JAB1 indeed correlated with reduced p27 levels (Figure 7A, B, and C) as well as Herceptin resistance in Herceptin resistant patient samples (Figure 7D). These exciting results prove that JAB1 is a potential biomarker for Herceptin resistance. Additionally, because inhibition of JAB1 had such a strong inhibitory effect on cell growth and motility that sensitized resistant cells to Herceptin, inhibition of JAB1 could be an attractive target for drug development in breast cancer.

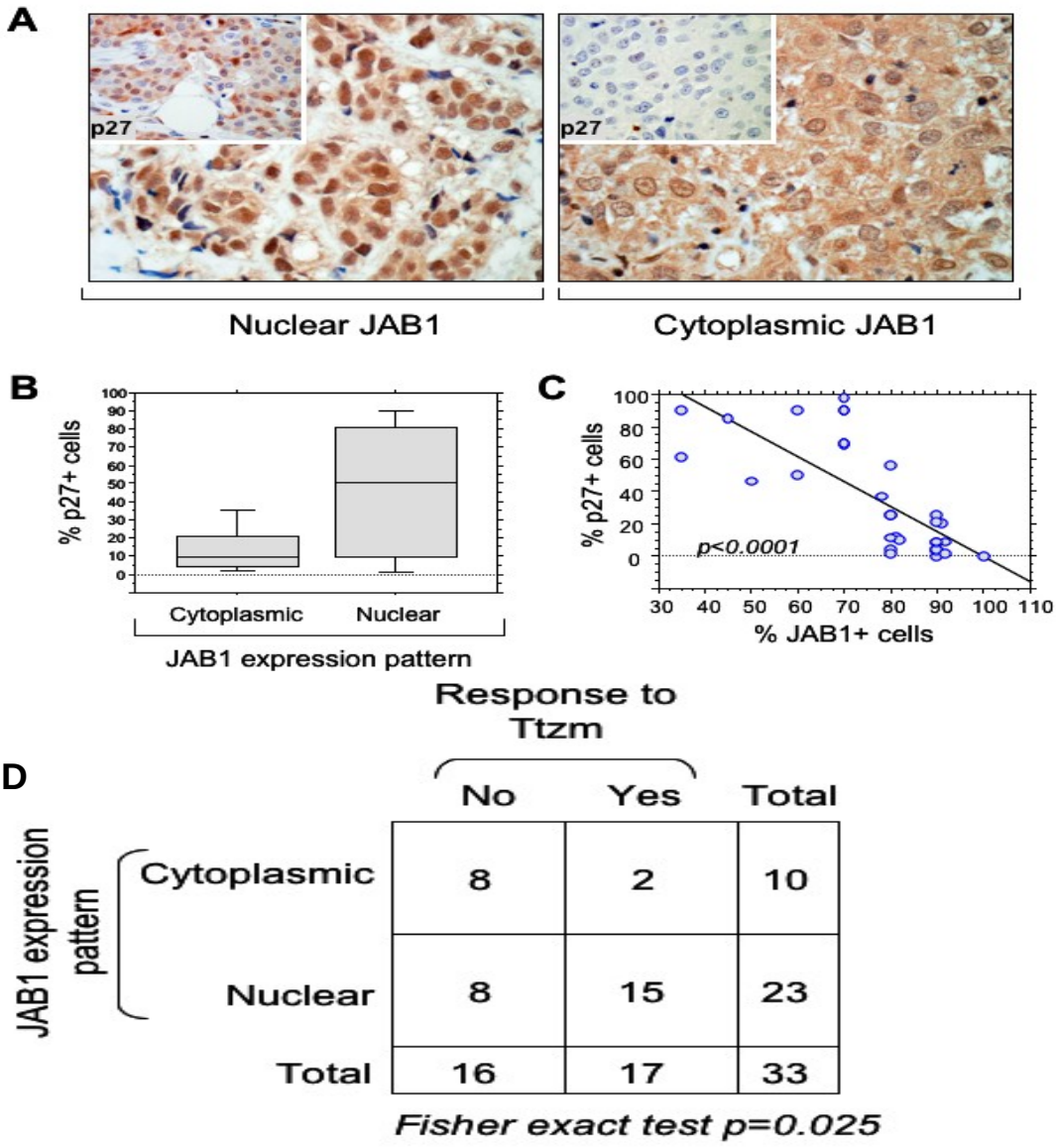


**Figure 7.** *JAB1 expression pattern in primary breast carcinomas predicted response to treatment with trastuzumab*. (A) Immunohistochemical staining of JAB1 in primary breast carcinomas revealed two different patterns, a predominantly nuclear (left) and a predominantly cytoplasmic (right). The corresponding p27 immunohistochemical expression is shown in the insets. (B) Predominantly cytoplasmic JAB1 expression pattern was correlated with lower p27 expression levels (Mann-Whitney U test, p=0.026) (C) Also the immunohistochemical JAB1 expression levels were inversely correlated with p27 expression levels (Spearman R=-0.78, p=0.0001). (D) Predominantly cytoplasmic JAB1 expression levels were correlated with resistance to treatment with trastuzumab (Fisher exact test p=0.025)

**Key Research Accomplishments**

Task 1A. We have identified the region that is essential for JAB1 transcription

Task 1B. We have identified key transcription factor binding elements in this region, C/EBP and

GATA1

Task 1C. We have confirmed binding of these transcription factors to these elements through EMSAs and ChIP. Additionally, we have found that STAT3 can bind to the promoter and compete with C/EBP beta 2 for binding. Further, that SRC activation of STAT3 is important for JAB1 transcripition.

Task 3A. We have shown that overexpression of JAB1 inhibits Herceptin treatment.

Task 3B. We have demonstrated that overexpression of JAB1 reduces Herceptin mediated G1 arrest and p27 accumultion in BT474 and SKBR3 sensitive cells. Also, that inhibition of JAB1 sensitizes breast cancer cells to Herceptin mediated p27 accumulation, G1 arrest and growth arrest.

Task 3C. In human breast cancer patient samples, high JAB1 cytoplasmic expression correlated with reduced p27 expression and with poor response to Herceptin.

**Reportable Outcomes**

This work has been presented at five conferences and the manuscripts are in preparation.

**Conclusions**

We have identified two transcription factors that bind the JAB1 promoter leading to an increase in its promoter activity. Further studies will be performed to determine which signaling pathway is responsible for this activation. Also, we have shown that JAB1 overexpression can confer resistance to Herceptin treatment and that inhibition of JAB1 by shRNA sensitizes cells to Herceptin treatment. Our data suggest that JAB1 could be an important prognostic marker for Herceptin resistance and an important therapeutic target to overcome Herceptin resistance.

**Absracts**:

1. Shackleford, T.J., Zhang, Q., Korapati, A., Tian, L., and Claret, F-X. Inhibition of JAB1 Reverses Resistance to Trastuzumab by Increasing the Stability of p27. *The Department of Defense Breast Cancer Research Program Fifth Era of Hope Meeting,* June 25-28, 2008, Baltimore, MD, **U.S.**

2. Shackleford, T.J., Zhang, Q., Korapati, A., Tian, L., Kute, T., and Claret, F-X.. Role of JAB1 in Resistance to Trastuzumab (Herceptin) Treatment. *AACR International Conference on Molecular Targets and Cancer Therapeutics,* October 22-26, 2007, San Francisco, CA, **U.S.** #B125.

3. Shackleford-Johnson, T.J., Tian, L, Korapati A, Zhang Q, Kute T, Claret, F-X.
Role of JAB1 in Resistance to Trastuzumab (Herceptin) Treatment.
*The 25th Congress of International Association for Breast Cancer Research,*
September 15-18, McGill University Cancer Center, Montreal, QC, **Canada**. #SaP38

4. Shackleford-Johnson, T.J, Tian L, Korapati A, Zhang Q, Kute T, Claret, F-X.
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*98th American Association for Cancer Research, Annual Meeting,*
April 14-18, Los Angeles, CA, **U.S.** #07-AB-4438

5. Shackleford-Johnson, T.J, Tian L, Korapati A, Zhang Q, Kute T, Claret, F-X.
Role of JAB1 in Resistance to Trastuzumab (Herceptin) Treatment.
Trainee and Recognition Day, MD Anderson Cancer Center
May 25, Houston, TX, **U.S.**

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Shackleford-Johnson, T.J., Korapati, A., Zhang, Q., Tian, L., and Claret FX.
Regulation of *Jab*1 gene transcription by C/EBP and STAT3 in breast cancer cells.
**Journal of Biological Chemistry** *In preparation*

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JAB1 Confers Trastuzumab Resisstance by Nuclear Transport of p27
**Cancer Cell**, *In preparation*